GENETICAL DETERMINANTS OF ZINC UPTAKE ASSOCIATED WITH CITROBACTER RODENTIUM PATHOGENESIS

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ABSTRACT

The human enteric pathogens Enteropathogenic *Escherichia coli* (EPEC) and Enterohemorrhagic *E. coli* (EHEC) are A/E pathogens that colonize in human gastrointestinal mucosa and cause foodborne diseases worldwide. *Citrobacter rodentium* (CR), the murine equivalent of EPEC and EHEC, shares similar pathogenic strategies and common virulence proteins with them. While A/E pathogens cause mild and self-limited infection in immunocompetent hosts, severe symptoms and high mortality have occurred in immunocompromised hosts. Taking advantage of mice genetics, CR infection in mice becomes a powerful small-animal model to study bacterial pathogenic mechanisms of A/E pathogens. Zinc homeostasis has long been regarded crucial in host-pathogen interactions, highlighting a key role of fine-tuned zinc uptake system for bacterial proliferation and virulence. However, a full-spectrum of factors related to zinc homeostasis regulation during CR infection remains elusive. We performed an unbiased screening after zinc depletion with a library of randomly mutated CR strains, revealing that interruption of *tolQ* or *recC* can result in growth defects of CR under zinc deficiency. Then we infected IL-22 knockout (*Il22*$^{-/-}$) mice with the transposon-interrupted and chromosomal knockout CR strains of *tolQ* and *recC*. Absence of functional TolQ or RecC in CR failed to reduce significant weight loss and high mortality in infected mice. Therefore, our results demonstrate that *tolQ* and *recC* are not genetical determinants of zinc-dependent pathogenicity during CR infection in *Il22*$^{-/-}$ mice, although they are indispensable for CR growth under zinc starvation conditions *in vitro*.

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Introduction

1. A/E pathogens EPEC, EHEC and *C. rodentium*

The human pathogens enterohemorrhagic *Escherichia coli* (EHEC) and enteropathogenic *E. coli* (EPEC) are the leading causative agents for foodborne diseases, which result in severe morbidity and mortality throughout the world [1]. EHEC, EPEC and the mouse-restricted pathogen *Citrobacter rodentium* (CR) colonize the intestinal mucosa via forming distinctive attaching and effacing (A/E) lesions at mucosal surface of host intestines, which are characterized by the attachment of the bacteria to epithelial cells, the destruction of brush border and microvilli, the formation of pedestal-like structures beneath the adherent bacteria through the rearrangement of actin, and the loss of ions, causing severe diarrhea [2-6,15,19]. As the murine equivalent of EHEC and EPEC, CR shares 67% of its genes, many common virulence factors and most pathogenic mechanisms with them [7]. They all possess a gene cluster locus required for A/E pathogenicity, the locus of enterocyte effacement (LEE) island, which encodes for the apparatus of type III secretion system (T3SS), the secreted effector proteins, as well as the regulatory factors of LEE gene expression [8,15]. However, the genes functionally related to A/E pathogenicity are only a small portion of the encoded genes in each genome of EHEC, EPEC, and CR [9]. Therefore, it is believed that the virulence regulation of A/E pathogens during their adaption to host gastrointestinal tract, the detailed mechanisms of A/E pathogen-induced intestinal disorders such as colitis and colon tumorigenesis, and how A/E pathogen infection contributes to host morbidity and mortality remain largely unknown, especially in

immunocompromised and/or immunodeficient settings [1,7].

2. Use *C. rodentium* infection in mice as a model to study A/E pathogenesis

CR infection in mice has become a powerful small-animal model to study the bacterial virulence mechanisms, host immune responses and pathogen–host–microbiota interactions during A/E pathogen infection, providing novel insights into enteric infections for us [10,17]. As EPEC and EHEC infections always lead to strikingly elevated morbidity and mortality in immunocompromised or immunosuppressed patients, the severity of CR infection is influenced by the host immunity conditions. The genetic background and the intestinal microbiota composition of different inbred mice strains have been recognized as key factors that contribute to distinct susceptibility and subsequent immune response to CR infection [11,12,13,14]. The infection of CR in wild-type C57BL/6 and many immunocompetent mouse strains normally develop mild symptom and self-limiting inflammation of the caecum and colon, showing little or no mortality, and the infection cycle be divided into four defined phases including the cecal establishment phase, the colonic expansion phase, the steady-state shedding phase and the clearance phase [10,13,16,17]. However, infection is fatal in some mouse strains with immune defects and would eventually reach host decline phase instead of clearance, during which the health status of the infected host rapidly deteriorates [18]. Interleukin-22 knockout (*Il22*$^{-/-}$) mice display dramatically increased intestinal epithelial damage, systemic bacterial burden and mortality during CR infection [20]. IL-22 is a cytokine preferentially produced by T helper type 17 (T_H17) cells, and the

receptor of IL-22 (IL-22R) is expressed specifically on epithelial tissues [21,22]. It is believed that IL22 plays a critical role in the early phase of host defense against A/E bacterial pathogens by protecting colonic epithelial integrity and mediating epithelial innate immunity [20,23]. Many studies have manifested that immunodeficient hosts are more susceptible to A/E pathogen-induced infections than healthy controls with normal IL-22 levels [20,24]. To explore novel virulence factors and molecular regulation mechanisms related to A/E pathogenesis, *Il22*$^{-/-}$ mice are used to achieve immunocompromised conditions in this study.

3. Metal homeostasis, nutritional immunity and zinc regulation system

Transition metals are necessary for the survival of all living organisms via participating in plenty of crucial biological processes, such as signaling transduction, transcriptional regulation, electron transfer, and oxidative stress response. The levels of nutrient metals must be carefully controlled to avoid toxicity or deficiency in cells [26,29,30,31], and the roles of these metals at the pathogen–host interface are very complicated. Firstly, dysregulation of host metal homeostasis due to genetic and dietary intake or absorption of metals could alter host's susceptibility to infections [30,32]. Besides, to prevent infection with pathogenic organisms, mammalian hosts have evolved a process termed nutritional immunity to limit the availability of metal nutrients at target sites, including activating epithelial cells and recruiting neutrophils, which could release antimicrobial metal-binding proteins like calprotectin at sites of infection [26,38]. In addition to mechanisms of withholding essential metals from invading bacteria, in

mammals, nutritional immunity kills bacteria by utilizing the toxic properties of transition metals [28]. Nutritional immunity is one of the most important host strategies to restrict the growth and reduce the virulence of pathogens. In turn, successfully adapted microorganisms have developed a number of sophisticated systems to overcome the limitations and acquire the metal ions indispensable for their survival [26,29]. It is generally accepted that exploring detailed mechanisms of metal acquisition strategies used by pathogens could be clinically significant to uncover new therapeutic targets [30,31].

In the vast majority of living systems, Zinc is the second most abundant transition metal, serving structural, regulatory and catalytic roles [27]. In prokaryotic organisms, almost 5-6% of all encoded proteins contain recognizable zinc-binding sites, and this proportion is even larger regarding eukaryotic proteome [26,29]. Because of such essential roles of zinc in both host and bacterial physiology, zinc-related pathogenic mechanisms and host responses should be critical of infectious diseases. While the mammalian hosts using various strategies against invading bacteria like zinc sequestration at mucosal and epithelial surfaces by multiple chelation processes, pathogens have acquired fine-tuned regulation systems to sense the changes in zinc concentration and make accurate response to adapt zinc-limiting conditions in the host. Zinc homeostasis in most bacteria is usually controlled through transcriptional regulators that repress gene expression through binding target DNA in the presence of zinc, whereas de-repress their regulons in the absence of zinc [29,33]. ZnuABC zinc transporter, a high-affinity zinc uptake (Znu) system conserved in most Gram-negative

bacteria, such as *E. coli* and CR, is required for bacterial zinc homeostasis in intracellular environments and contributing to the virulence of pathogens [34]. Zinc uptake regulator (Zur), a member of the Fur family of metal-responsive regulators, is in charge of the transcription of genes encoding for the components of ZnuABC uptake system——the ZnuB channel, the ZnuC ATPase component which provides the energy necessary for zinc import, and ZnuA, a solute-binding protein that captures Zn^{2+} in the periplasm with high efficiency and delivers it to ZnuB [35,36,37]. A series of studies carried in bacterial species which depend on ZnuABC to infect their hosts have established that disruption of *znuABC* genes not only impair their ability to growth in *in vitro* environments poor of zinc, but also lead to a remarkable loss of pathogenicity [37]. Taken together, sequestration of zinc is an important defense strategy for the host to restrict bacterial infection. In response to the limiting concentration of zinc in the intestinal tract, pathogens with a well-developed zinc homeostasis control mechanism can neutralize the stress and even exploit this harsh condition to gain competitive advantages over the gut microbiome [38,39]. Although bacterial zinc transport systems have been characterized for their relevance to virulence, detailed regulation mechanisms and additional factors of this pathway during bacterial infection remain poorly understood.

To explore underlying genetical determinants of zinc uptake associated with CR pathogenesis, we generated a single-gene mutant library via Tn5 transposon-mediated random mutagenesis and performed a high throughput screening under zinc starvation *in vitro*. Through following infecting $IL22^{-/-}$ mice with wild-type CR or

genetically manipulated mutant strains, we revealed that although interruption of *tolQ* or *recC* can result in growth defect of CR under zinc deficiency *in vitro*, they do not participate in the zinc transport pathway in response to zinc shortage *in vivo*, which means they are not CR virulence-related genes.

Materials and Methods

1. Ethics Statement

All animal experiments were carrying out according to protocol number MO19H269, approved by the Johns Hopkins University's Animal Care and Use Committee. C57BL/6-background IL-22 knockout (*Il22*$^{-/-}$) mice were maintained in a specific pathogen-free facility and were given sterile food and water *ad libitum*.

2. Generation of *C. rodentium* mutant library

The mutant library of CR was generated via the Tn5 transposon-mediated random mutagenesis. All CR mutants were derived from wild-type CR (DBS 100 strain) and were mutagenized by using of EZ-Tn5™ <KAN-2> Tnp Transposome™ Kit (Lucigen Corporation, Madison, WI). To prepare the electrocompetent wild-type CR, 2 ml of bacteria culture was taken from the overnight CR culture and diluted in 100 ml LB broth medium for subculture at 37℃ with shaking at a speed of 250 rpm until the optical density at 600nm (OD_{600}) reached 0.4. Then the bacteria were washed by 40ml of ice-cold sterile H_2O for two times and 40 ml of chilled 10% glycerol for three times. Each wash cycle included centrifugation at 4,000 rpm for 15 minutes, removing of supernatant and resuspension of pellets. Then 10ng of EZ-Tn5 <KAN-2> Transposome were transferred into electrocompetent wild-type CR by electroporation, using the Ec1 program of a Bio-Rad MicroPulser (Bio-Rad Laboratories, Hercules, CA). The electroporated cells were gently mixed with 1 ml of SOC medium in the electroporation cuvette (USA Scientific, Ocala, FL) and then transferred to a tube. After

incubated in a 37°C shaker for 60 minutes for recovery, the bacteria were plated onto LB agar plates containing 30 μg/ml kanamycin and incubated overnight to select Tn5-inserted CR mutants that can express the Tn903 kanamycin resistance gene (Kan^R). To generate the library of CR mutants, each single colony was picked from the LB plates with kanamycin and inoculated into one well of a containing 100 μl/well LB broth by an autoclaved toothpick. After overnight statically culture at room temperature, 100 μl of 50% glycerol was added into each well. Then the 96-well plates harboring CR mutants were stored in -80°C freezer for screening.

3. Screening of *C. rodentium* mutants with growth defect under zinc deficiency

An unbiased screening under zinc starvation condition was performed on the Tn5-insertion mutant CR library. *N,N,N′,N′*-tetrakis-(2-pyridylmethyl)ethylenediamine (TPEN), a membrane-permeable chelator with high affinity to Zinc, was added into LB agar plates to create a zinc-deficient condition for bacterial growth. Taken from storage at -80°C and thawed fully at room temperature, CR mutants in the 96-well plates were inoculated by a 96-pin Replica plater (Sigma-Aldrich, St. Louis, MO) onto LB plates containing 20 μM of TPEN. The plates were incubated at 37°C overnight. Compared to wild-type CR, served as negative control, and *ΔznuA* CR, served as positive control, the Tn5-interrupted CR mutants showing significant growth defects under zinc-starved condition were selected from the library.

4. Identification of Tn5 insertion sites in mutant *C. rodentium* strains

The transposon insertion site in the genome of each candidate CR strain was identified by a two-step arbitrary PCR followed by Sanger sequencing. To prepare the template for PCR reaction, 1 ml of overnight cultured CR was spun down by centrifugation at 8,000 rpm for 3 minutes and washed with 1 ml H_2O for two times. The resuspended bacterial cells were submerged in 1 ml H_2O and then boiled at 95°C for 10 minutes. After centrifugation at 12,000 rpm for 1 minute, 5 µl of supernatant was used as template for the arbitrarily primed PCR reaction. The details of primers used in this reaction are in **Table 1** and the reaction system is shown in **Table 2**. The PCR products purified by Lipuret™ Gel Extraction Kit (LifeSct, Rockville, MD) were sent to Sanger sequencing using Tn5_Round3 primer. The sequencing results were aligned to CR genome by SnapGene software version 4.1.9 (from Insightful Science; available at snapgene.com) to determine the transposon insertion site in CR genome.

5. Generation of gene knockout *C. rodentium* strains

To rule out the possible off-target effect during transposon insertion, single gene knockout mutant CR strains were generated by a scarless in-frame gene deletion strategy. The suicide plasmid pRE112 was used as a vector to construct GOI knockout plasmids, which was integrated into CR genome via homologous recombination and induced a *sacB*-based allelic exchange [1]. To prepare the empty vector, pRE112 plasmid was linearized by restriction digestion by KpnI and SacI (New England BioLabs, Ipswich, MA). 800-1100 bp upstream and downstream fragments of the target gene were amplified by PCR using super-fidelity SuFi DNA polymerase (LifeSct, Rockville, MD)

using the primers as detailed in **Table1** and the reaction system detailed in **Table 3**. The purified PCR products and pRE112 backbone were ligated together using Gibson Assembly Kit (New England Biolabs, Ipswich, MA), and the mole ratio of each insert fragment to vector is 2:1 in this study. After incubation at 50°C for 1.5 hours, 2 µl of the Gibson reaction product was electroporated into 50 µl of electrocompetent SY327 cells. After recovery in 500 µl SOC medium at 37°C with shaking for 60 minutes, all electroporated cells were plated onto a LB agar plate containing 25 µg/ml chloramphenicol (Cm) and incubated at 37°C overnight. SY327 bearing engineered plasmid acquired resistance to chloramphenicol (Cm^R). Single clones were picked out and cultured overnight in 5 ml LB broth medium with chloramphenicol, and the pRE112-GOI-KO plasmid was extracted by mini-preparation using a LiPure™ Plasmid Mini Kit (LifeSct, Rockville, MD) and confirmed through PCR followed by Sanger Sequencing using the check primers with detailed sequences shown in **Table1**. To generate pRE112-GOI-KO plasmid-harboring SM10 cells to serve as donor strain for conjugation, 100 ng plasmid was transferred into 100 µl of chemical-competent SM10 cells by heat shock at 42°C for 45 seconds. The recipient strain wild-type CR containing ampicillin-resistant (Amp^R) pKD46 plasmid was mixed with SM10 donor cells containing chloramphenicol-resistant (Cm^R) pRE112-GOI-KO plasmid and incubated together on a LB agar plate at 30°C for 24 hours. After conjugation, successfully conjugated clones with both Cm^R and Amp^R were selected by LB plates added 100 µg/ml ampicillin and 25 µg/ml chloramphenicol and then statically grow in 1 ml of LB-NaCl at 30°C incubator for 7 hours. Diluted bacteria culture was plated onto LB- NaCl+

5% sucrose plates and incubated at room temperature for 2 days. The replacement of the original allele by the recombinant allele was facilitated by homology and the loss of integrated plasmids were accomplished by the expression of *sacB*, which was toxic in the presence of 5% sucrose. Single clones were picked and streaked onto a LB-ampicillin plate and a LB-chloramphenicol plate. After overnight growth, the recombined CR strains with Amp^R but not Cm^R were selected as potential candidates. Confirmation PCR was carried out with the primers detailed in **Table 1** and the conditions detailed in **Table 4**. The verified knockout strains were then cultured at 42°C to get rid of temperature-sensitive pKD46 plasmid, thus scarless single-gene knockout strains of CR were obtained.

6. Growth curve measurement of *C. rodentium* strains

For growth curve measurement, the overnight CR cultures were diluted at 1:100 after normalizing OD_{600} to 1.0 and grown in LB broth medium at 37°C with shaking at a speed of 250 rpm. 1 ml of each CR culture was taken at indicated time periods to read OD_{600} with the use of a CO8000 Cell Density Meter (Denville Scientific, Metuchen, NJ).

7. *C. rodentium* infection in mice and statistical analysis

Overnight CR cultures were normalized OD_{600} to 1.0 before concentrating by 10 times in 1x PBS solution. After a 6-hour fast, male *Il22*$^{-/-}$ mice (3 to 6 months) were orally inoculated with 200 µl of PBS containing indicated CR strains. The body weight, diarrhea severity, and morbidity of mice were observed every two days. For fecal CR

burden analysis, stool samples were collected from live animals at indicated days post inoculation. The fecal samples were homogenized in PBS solution based on the stool weight at a ratio of 10 mg in 100 µl. After serial dilutions from 10^{-1} to 10^{-5}, a 10 µl aliquot from each of the 10^{-5} to 10^{-1} dilutions was dotted on MacConkey agar plates. The plates were put into 37°C incubator and the Colony Forming Units (CFUs) of CR were enumerated the following day.

All statistical analysis was performed using GraphPad Prism version 8.0.2 (GraphPad Software, San Diego, CA). Standard errors of means (S.E.M.) were plotted in graphs. Statistical analysis on survival curves was carrying out using the log-rank (Mantel-Cox) test. Student's t test was applied for statistical analysis on relative body weight change. Significant differences were considered: ns, non-significant difference; * at $p < 0.05$; ** at $p < 0.01$; *** at $p < 0.001$; **** at $p < 0.0001$.

Primers	**Sequences**
Tn5_Round1	5’-TCATTGGCAACGCTACCTTTG-3’
AH-Tn5_Round1	5’-GGCCACGCGTCGACTAGTACNNNNNNNN-3’
Tn5_Round2	5’-ATTATCGCGAGCCCATTTATACC-3’
AH-Tn5_Round2	5’-GGCCACGCGTCGACTAGTAC-3’
Tn5_Round3	5’-GACGTTTCCCGTTGAATATGGC-3’
tolQ_up1000_F	5’-caagcttcttctagaggtaccAGGCAAGCAAAGCCGGTC-3’
tolQ_up1000_R	5’-tcgccatggcTGCTTAAACTCCGCGACAATAG-3’
tolQ_dw800_F	5’-agtttaagcaGCCATGGCGAGATCGCGTG-3’
tolQ_dw800_R	5’-catgaattcccgggagagctcGCGTTCCTGTTCCGCCGC-3’
recC_up1100_F	5’-caagcttcttctagaggtaccGCGCGCCGGGTACTGTTA-3’
recC_up1100_R	5’-aatttttatacaaGGAGACTCCTGACAACTAACGG-3’
recC_dw900_F	5’-aggagtctccTTGTATAAAAATTGCGCATC-3’
recC_dw900_R	5’-catgaattcccgggagagctcCCGGCTTTTTGATATTTTTG-3’
tolQ_Up239F	5’-gtggccttcgtggtgcgtaaaatg-3’
tolQ_Gene181R	5’-cgataccggaccagaatttgtct-3’
tolQ_Dw345R	5’-gtctttcgcgccaccgatcaa-3’
recC_Up274F	5’-gctgatggtgatggtggtcac-3’
recC_Gene153R	5’-cgacagggtcatctgtagccac-3’
recC_Dw313R	5’-gagagagactttaccgcctgc-3’

Table1. PCR primer sequences

1st Round PCR Reagents	**Volume (µl)**
MyTaq™ Red Mix, 2× (Bioline)	10
Template	5
Round 1 Primers Mix	1
Sterile H_2O	4
Total	**20**
(1) 95°C 5 minutes (2) 94°C 30 seconds (3) 42°C 30 seconds (4) 72°C 3 minutes **Go to (2) for 6 times** (5) 94°C 30 seconds (6) 52°C 30 seconds (7) 72°C 3 minutes **Go to (5) for 25 times**	
2nd Round PCR Reagents	**Volume (µl)**
MyTaq™ Red Mix, 2× (Bioline)	50
Template (Add 80µl H_2O to 1st round product)	5
Round 2 Primers Mix	10
Sterile H_2O	35
Total	**100**
(1) 95°C 5 minutes (2) 94°C 30 seconds (3) 42°C 30 seconds (4) 72°C 3 minutes **Go to (2) for 40 times**	

Table2. Arbitrary PCR systems

Reagents	Volume (μl)
2x LiSpark™ Max Buffer	12.5
dNTP Mix (10mM each)	0,.5
Template DNA	1
Forward primer	1
Reverse primer	1
2x LiSpark™ Max SuFi DNA Polymerase	0.5
Sterile H_2O	8.5
Total	**25**
(1) 95°C 5 minutes (2) 95°C 15 seconds (3) 55-70°C 15 seconds (4) 72°C 2 minutes **Go to (2) for 34 times** (5) 72°C 30 seconds	

Table3. PCR system for homologous arm amplification

Reagents	Volume（μl）
MyTaq™ Red Mix, 2× (Bioline)	10
Template	optional
Forward primer	1
Reverse primer	1
Sterile H_2O	optional
Total	**20**
(1) 95°C 8 minutes (2) 95°C 15 seconds (3) 56°C 15 seconds (4) 72°C 2 minutes **Go to (2) for 27 times** (5) 72°C 30 seconds	

Table4. PCR system for knockout confirm

Results

1. Unbiased screen of single-gene *C. rodentium* mutants with growth defects under zinc starvation

To systemically analyze the genes encoded in CR genome, we utilized a random mutagenesis strategy mediated by Tn5 transposon to randomly interrupt the gene individually in wild-type CR (DBS 100 strain). The EZ-Tn5 Transposon with Tn903 kanamycin resistance gene (Kan^R) would randomly insert into genomic DNA of CR after the activation of Tn5 transposase by magnesian ions in the bacterial cell. The Tn5 transposon-inserted CR mutants were thus screened through LB-kanamycin plates.

To explore the genetic determinants of zinc uptake system in CR under a zinc-deficient environment, the Tn5 mutation CR library was grown on LB agar plates added TPEN, which can grab surrounding zinc ions. Mutants with growth defects under zinc depletion were visually identified and validated. After that, the Tn5 transposon insertion site in each individual mutant was mapped to the genome of CR. By the end of this study, a single-gene-mutant library consisting of approximately 30,000 Tn5-interrupted CR mutant strains was generated and divided into 17 sub-libraries. Through our unbiased screen, 20 mutant CR strains exhibiting significant growth defects under zinc deficiency have been identified so far.

2. Identification of TolQ and RecC as factors involved in *C. rodentium* zinc uptake system

The arbitrary PCR products of two of our identified Tn5 mutants from sub-library 13

and 14 were sequenced and the results were mapped to CR genome. We identified that the Tn5 interruption site in Lib13 C4-F7 strain was at *tolQ* gene, which truncate the its ORF at 27th amino acid. And the insertion site in Lib14 B5-H10 strain was at *recC* gene, truncating *recC* ORF at 147th amino acid **(Figure 1A and 1B)**. Indeed, the Tn5 transposon interruption caused major truncations in TolQ and RecC proteins, which could substantially impair their functions. After normalizing OD_{600} to 1.0, the growth of these two mutants was as normal as WT and *ΔznuA* controls in medium that contains sufficient zinc, demonstrating that interruption of *tolQ* or *recC* gene does not impact the proliferation of CR in rich medium. On the contrary, the growth of these two mutant strains was affected greatly in the presence of 20 μM TPEM, suggesting that *recC* gene-encoded RecC protein and *tolQ* gene-encoded TolQ protein could independently play an important role in the zinc uptake system for CR response to low zinc environmental stress.

3. The absence of functional TolQ or RecC failed to attenuate *C. rodentium* virulence in *Il22*$^{-/-}$ mice

To eliminate possible off-target effect by Tn5 transposon insertion, a *Δrec* strain and a *ΔtolQ* strain, in which the *recC* gene and the *tolQ* gene was respectively deleted from CR genome. The PCR-amplified 0.8-1.1 kbp upstream and downstream fragments of each target gene **(Figure 2B)** and the linearized pRE112 backbone were ligated together by Gibson assembly to construct a pRE112-*recC*-KO plasmid and a pRE112-*tolQ*-KO plasmid **(Figure 2A)**. The plasmids were extracted out from SY327 and were

verified by PCR using indicated check primers **(Figure 3A and 3B)**. WT CR were subjected to conjugation with SM10 donor cell carrying pRE112-*recC*-KO plasmid and a pRE112-tolQ-KO plasmid and the successfully recombined CR expressing both Cm^R and Amp^R were selected. After sacB-based allelic exchange, candidates only with resistance to ampicillin were picked for PCR confirmation using two pairs of check primers **(Figure 3C and 4A)**. The growth dynamics of WT, *tolQ*::Tn5, *recC*::Tn5, Δ*recC* and Δ*tolQ* CR were monitored in LB medium, in order to examine whether RecC or TolQ impacts bacterial proliferation. Indeed, all the above mutant CR strains exhibited no defect in bacterial growth in LB culture, compared to WT CR **(Figure 4B)**.

To assess the impact of TolQ and RecC on CR virulence, we then carried out in vivo infection assays. *Il22*$^{-/-}$ mice were infected with wild-type, *tolQ*::Tn5, *recC*::Tn5, Δ*recC* and Δ*tolQ* CR strains. Clinical manifestations, body weight change, and fecal CR CFU were monitored after inoculation. We examined the colonization and proliferation abilities of WT CR and mutant strains in the colon of infected *Il22*$^{-/-}$ mice by counting viable CR recovered from the fecal samples [1]. The CFUs of WT, *tolQ*::Tn5, *recC*::Tn5, Δ*recC* and Δ*tolQ* CR in stool derived from infected mice at 4 and 7 dpi were almost identical **(Figure 5B)**. Therefore, Interruption and deletion of *tolQ* or *recC* did not lead to a decrease in CR burden in the infected *Il22*$^{-/-}$mice in vivo. All five groups of *Il22*$^{-/-}$ mice developed typical CR infection symptoms including diarrhea, dehydration and hematochezia. Besides, Infection with all five CR strains caused similar substantial body weight loss and severe lethality in infected mice within 16 days post inoculation (dpi). There were no significant differences in relative weight loss and survival rate

between mice inoculated with WT CR and other four CR mutants **(Figure 5A and 5C)**. Together these results indicate that the absence of functional TolQ or RecC failed to attenuate CR virulence in *Il22*$^{-/-}$ mice. Although *tolQ* and *recC* may play roles in CR zinc homeostasis regulation under *in-vitro* zinc depletion conditions, they are not genetical determinants of zinc uptake associated with CR pathogenesis.

Discussion

EPEC and EHEC are A/E pathogens with pathogenic capacity to cause gastrointestinal infections and result in significant diarrheal and extraintestinal diseases, imposing significant economic burdens and remaining major public health issues [19]. Improving our understanding of the pathogenic mechanisms of EPEC and EHEC will greatly facilitate new discoveries in treatment against EPEC and EHEC infections in humans [1]. Due to the fact that mice are naturally resistant to infections caused by EPEC and EHEC, infection in mice with CR, the murine equivalent of EPEC and EHEC, becomes a potent small animal model to investigate the A/E pathogenesis. Compared to wild-type C57BL/6 mouse strain, the significantly severer disease and higher mortality are triggered by CR infection in *Il22*$^{-/-}$ mice [20]. Meanwhile, it has been revealed that EPEC and EHEC are leading causes of lethality associated with diarrhea among children under age of 5 years, a period in which the low circulating IL-22 levels in host was defined as a critical risk factor for serious infections [19, 25]. The severe outcomes of A/E pathogen infections in immunocompromised and/or immunodeficient hosts is proved by both mice infection results and epidemiological studies. However, a substantial amount of underlying virulence mechanisms and pathogen-host interactions still remains elusive [7]. Transition metals like zinc, are essential nutrient substance to both hosts and invading pathogens. To adapt a zinc-deficient conditions generated by host, a fine-regulated zinc uptake system maintaining zinc homeostasis is of pivotal importance for bacterial proliferation and virulence. Uncovering the crucial virulence factors and a full-spectrum of molecular and cellular mechanisms related to zinc

homeostasis control under pathophysiological conditions would offer potential therapeutical target for A/E pathogen infections.

Here we generated a CR mutant library based on random mutagenesis for an unbiased screening of novel genetic determinants related to the zinc uptake regulation system in CR. To further investigate the possible relevance of the identified gene candidates to CR pathogenesis, *Il22*$^{-/-}$ mice were infected with WT and mutant CR strains. Our *in vitro* screening under zinc-chelated condition has revealed that interruption of *tolQ* and *recC* genes decreased the CR capacity to grow on LB plates containing TPEN. Thus, TolQ, a Tol-Pal system protein important in maintaining cell membrane integrity, and RecC, a subunit of RecBCD nuclease, are potential regulatory factors involved in CR zinc transport pathway [40]. However, the mice infection results showed that both Tn5 interruption and chromosomal deletion of *tolQ* and *recC* failed to impair the ability of proliferation, colonization and virulence of CR *in vivo* under pathophysiological conditions, suggesting that these genes are not genetical determinants of CR pathogenesis-related zinc uptake regulation system.

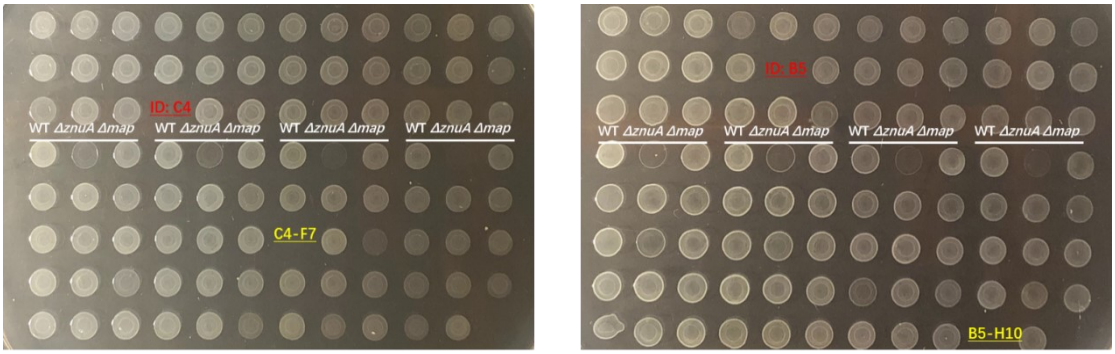
A possible explanation of our findings is that CR has developed redundant zinc uptake regulation pathways in response to zinc shortage *in vivo*. The factors related to zinc transport *in vitro* might lose their vital roles after CR adaption to the zinc chelation environment in host gut, which is much more complex than *in vitro* conditions because of host-pathogen-microbiota interactions that are absent in zinc-deficient conditions induced by TPEN. Some effector proteins indispensable for CR pathogenesis and with similar functions might functionally substitute RecC and TolQ or compensate the loss

of them during infection, participating in zinc acquisition through other regulation mechanism. Moreover, the IL-22 depletion in *Il22*$^{-/-}$ mice might explain our findings. In the host immune response against CR, IL-22 can induce the secretion of multiple antimicrobial proteins, including chelator proteins which can restrict metal availability of pathogens to reduce bacterial growth and virulence. Loss of IL22 leads to a decrease in host capacity of zinc sequestration, and CR might counteract the intestinal zinc shortage in IL-22 deficiency mice without utilizing the same zinc uptake mechanism as *in vitro*. Therefore, the interruption or knock out of *recC* and *tolQ* did not affect the pathogenicity of CR and failed to abolish the high morbidity and mortality rates in hosts.

In summary, our data shows that *recC* and *tolQ* are not genetical determinants of zinc uptake associated with CR pathogenesis. RecC and TolQ do get involved in zinc import pathway *in vitro*, but they play no critical roles related to CR virulence *in vivo* or their loss could be compensated during CR infection in *Il22*$^{-/-}$ hosts. More studies are needed to discover the mechanisms of zinc uptake regulation and relevant novel virulence factors of CR infection in immunocompromised hosts.

Figures and Figure Legends

A



B

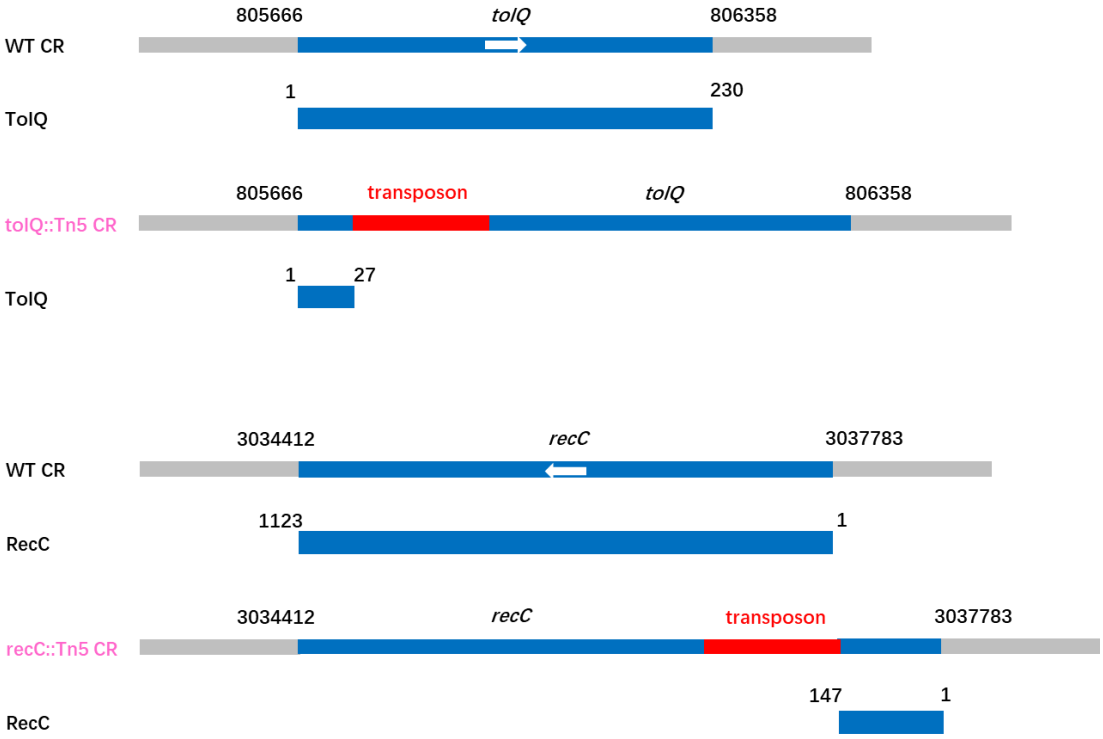


Figure1. The *tolQ::Tn5* and *recC::Tn5* CR mutants were identified during the screening. (A) Original screening plates (LB+20 μM TPEN) with potential promising Tn5-transposon-interrupted CR mutants (Lib13 C4-F7, Lib14 B5-H10). (B) Schematics of normal or Tn5-interrupted *tolQ* and *recC* and their protein expression in WT and mutant CR, respectively. The mutant genes and corresponding transposon insertion sites were identified through arbitrary PCR followed by Sanger Sequencing.

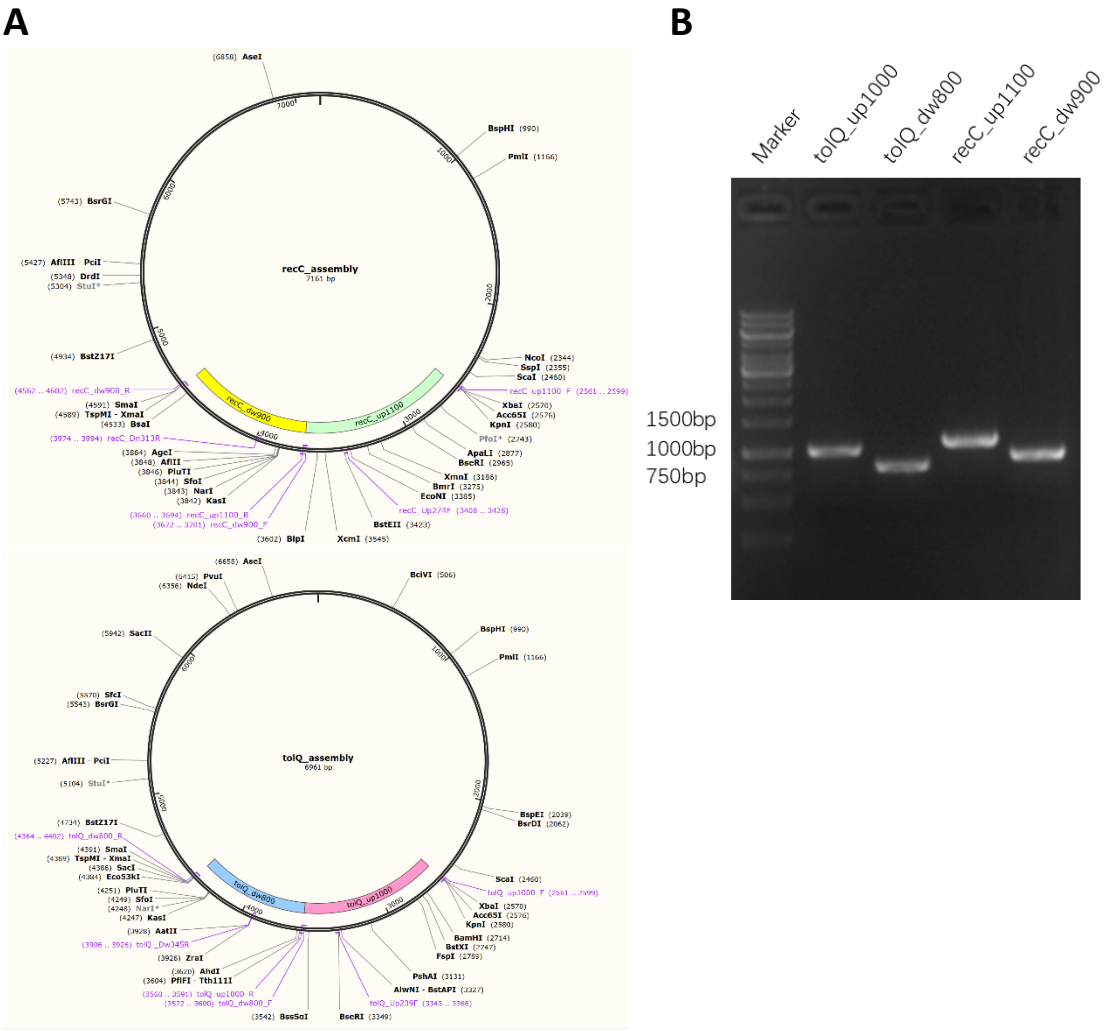


Figure2. The construction of chromosomal gene deletion plasmids. (A) Diagrams of pRE112-*recC*-KO plasmid and pRE112-*tolQ*-KO plasmid, containing 0.8-1.1 kbp upstream and downstream fragments of the target gene. (B) Image of amplified upstream and downstream homologous arms of *tolQ* and *recC*.

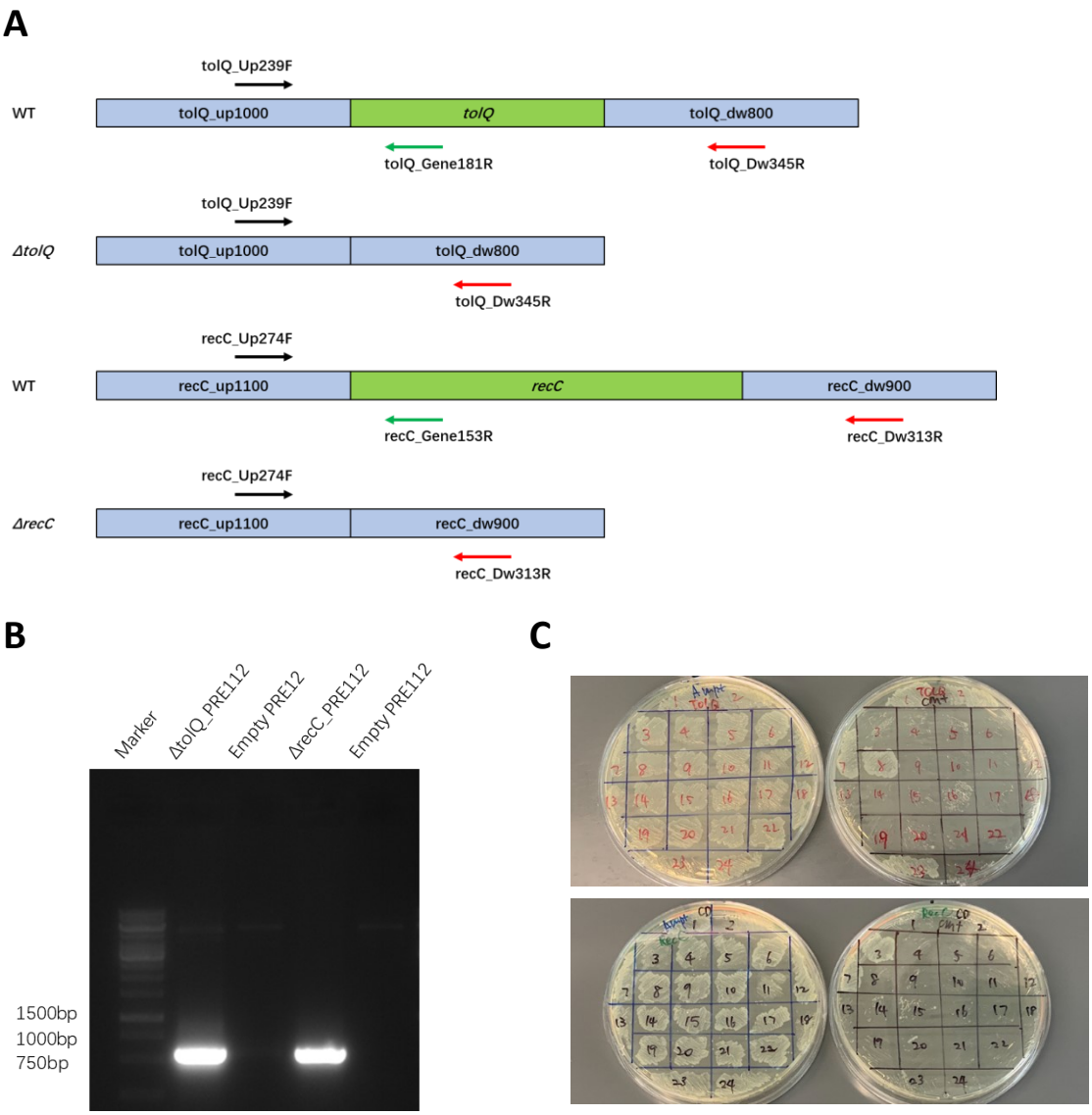
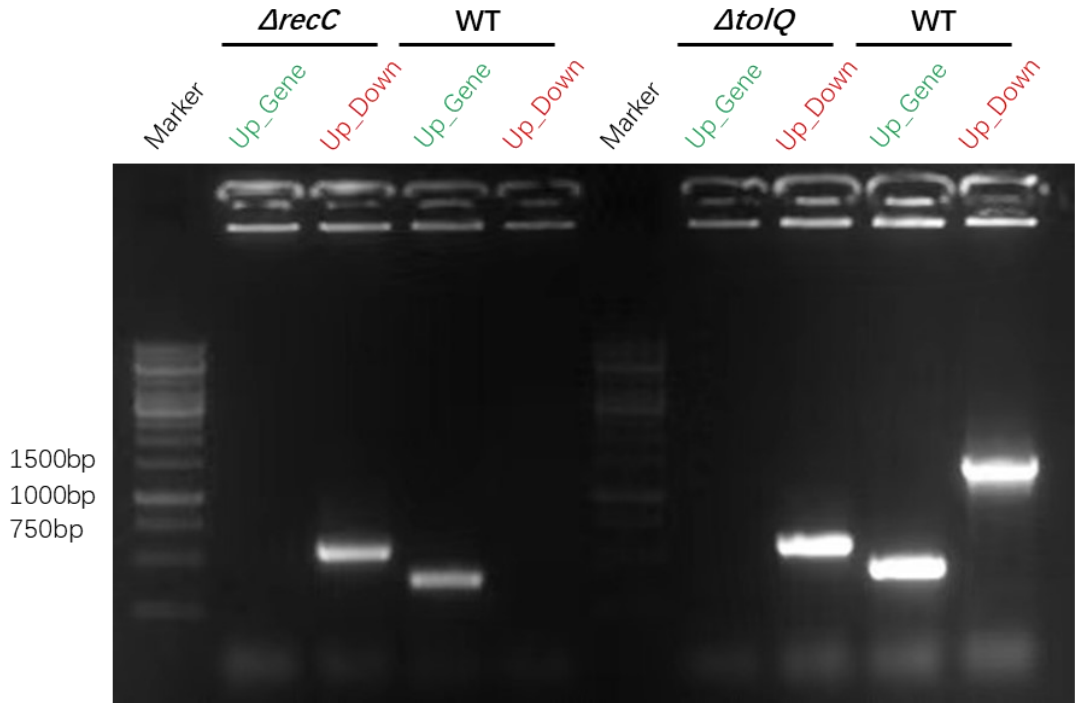


Figure3. In-frame knockout of *tolQ* and *recC* in CR via sacB-based allelic exchange. (A) Diagram of primer design for gene knockout confirmation. A common forward primer and a Gene reverse primer (in greed), or a Down reverse primer (in red) are used in PCR reaction. (B) PCR confirmation of recombinant plasmids using Up-Down pair of primer. Empty pRE112 plasmid served as a negative control. (C) Original screening plates on which recombined CR strains with Amp^R but no Cm^R were selected as potential candidates for further PCR validation.

A



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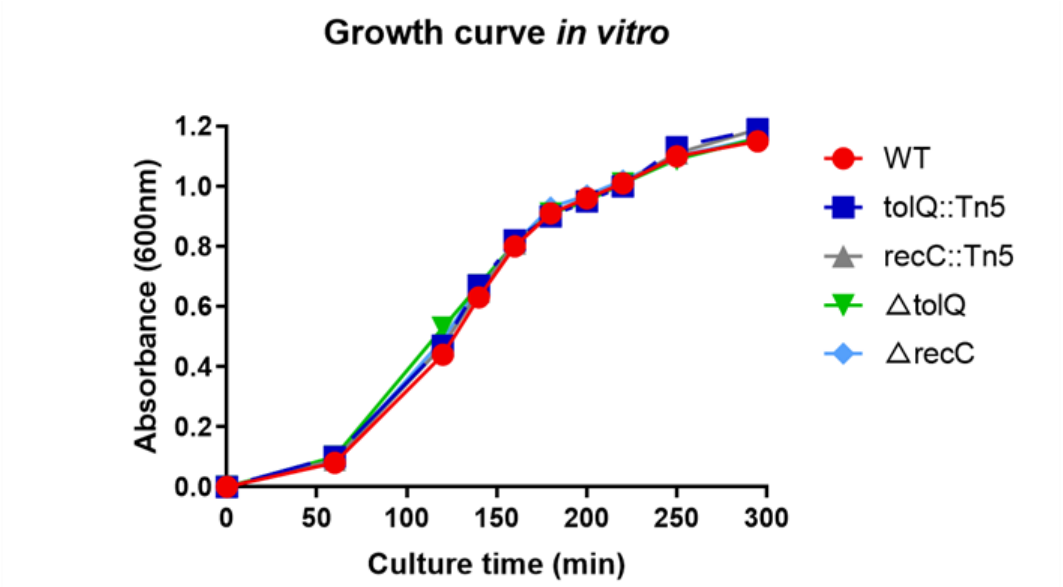

Figure4. Confirmation and growth curve measurement of mutant CR strains. (A) PCR confirmation of chromosomal gene deletion using Up-Gene and Up-Down primer pairs. WT CR was used as a negative control. In *Δrec* or *ΔtolQ* CR, no Up-Gene fragments was amplified due to the absence of target gene. While in WT CR, there could be no or a much larger sized Up-Down fragment amplified by PCR according to the target gene size and elongation time. (B) Growth curves of WT, *tolQ*::Tn5, *recC*::Tn5, *ΔrecC* and *ΔtolQ* CR strains in LB medium, at 1: 100 dilutions from the overnight cultures.

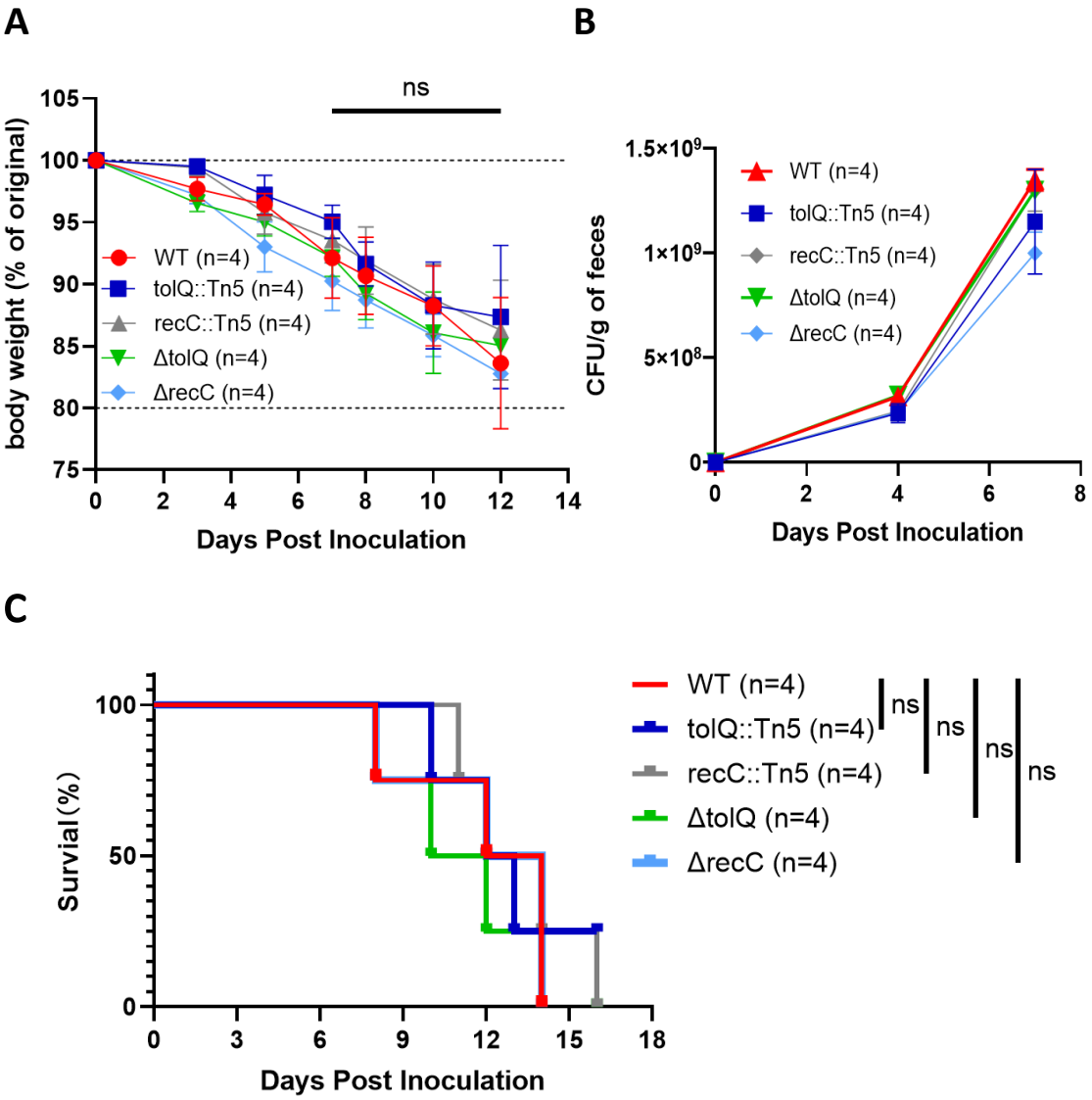

Figure5. Neither *recC* or *tolQ* is a genetical determinant for CR virulence in *Il22*$^{-/-}$ mice. (A) Body weight change of *Il22*$^{-/-}$ mice inoculated with WT, *tolQ*::Tn5, *recC*::Tn5, *Δrec* and *ΔtolQ* CR strains. (B) Fecal CR burden at indicated days post inoculation of WT or mutant CR strains in infected mice inoculated with 2 × 10^9 CFU of CR. CFUs of mutant CR strains exhibited no colonization defect in *Il22*$^{-/-}$ mice (C) Survival rate of *Il22*$^{-/-}$ mice inoculated with WT and mutant CR strains (ns, not significant; *p < 0.05, **p < 0.01, and **** p < 0.0001).

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Curriculum Vitae

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EDUCATION

Hunan University, School of Biology, Changsha, China *09/2015-06/2019*
Degree: Bachelor of Science
Major: Biotechnology
GPA:3.7

Johns Hopkins University, Bloomberg School of Public Health, Baltimore, MD, US *09/2019-present*
Degree: ScM (Master of Science)
Major: Biochemistry and Molecular Biology
GPA:4.0
Language: TOEFL 101 (Reading: 30 Listening: 27 Speaking: 20 Writing: 24) *10/2020*

HONORS & AWARDS

1st Comprehensive Scholarship, Hunan University, Changsha, China *2016*
2nd Comprehensive Scholarship, Hunan University, Changsha, China *2017*

RESEARCH EXPERIENCES

***Citrobacter rodentium* Pathogenesis in *Il22*$^{-/-}$ Mice** *11/2019-present*
Advisor: Prof. Fengyi Wan, Dept. of Biochemistry & Molecular Biology, JHSPH

- Constructed plasmids for scarless gene knockout in *C. rodentium*
- Generated 10 gene knockout *C. rodentium* strains
- Assessing the impacts of candidate gene deletions on *C. rodentium* infection caused mortality and clinical manifestation in infantile *Il22*$^{-/-}$ mice.

Generation of *C. rodentium* Mutant Library and Identification of Potential Virulence Genes for *C. rodentium* Infection in *Il22*$^{-/-}$ Mice *07/2020-present*
Advisor: Prof. Fengyi Wan, Dept. of Biochemistry & Molecular Biology, JHSPH

- Constructing a mutant *C. rodentium* library by Tn5 transposon random mutagenesis
- Screening for the essential genes related to zinc uptake system of *C. rodentium*
- Testing gene candidates in *Il22*$^{-/-}$ mice.

Separation and Purification of Virulence Proteins of A/E Pathogens for Mice Immunization and Antiserum Collection *09/2020-present*
Advisor: Prof. Fengyi Wan, Dept. of Biochemistry & Molecular Biology, JHSPH

- Constructed expression plasmids of virulence proteins and tested their expression
- Extracted and purified virulence proteins
- Conducted mice immunization for production of antiserum.

Undergraduate Thesis Project *09/2018-05/2019*
Molecular Cloning and Biochemical Function Analysis of Deubiquitinating Enzyme OTUD3

Advisor: Prof. Mao Ye, Molecular Science and Biomedicine Laboratory, State Key Laboratory of Chemo/Biosensing and Chemometrics, College of Life Sciences, Hunan University

- Constructed the OTUD3 eukaryotic expression plasmid and tested its expression
- Overexpressed OTUD3 in lung carcinoma cell A549 and detected the expression of several proteins
- Analyzed the effect of OTUD3 overexpression on A549 cell proliferation
- Hypothesized OTUD3 may act on cell cycle regulator p21 to inhibit the development of lung cancer.

Chengdu Institute of Biological Products Co., Ltd. Chengdu, China *07/2018*

Department of Vaccine Development

Intern

- Extracted and purified the *Streptococcus pneumoniae* capsular polysaccharide
- Combined the capsular polysaccharide with the tetanus toxoid
- Determined the free sugar content by sodium deoxycholic acid test, the antigen-antibody binding ability by ELISA, the protein content by Lowry method, the polysaccharide by anthrone-sulfuric acid colorimetry, and the residual amount of DMAP by HPLC, etc.

Chengdu Suncadia Medicine Co., Ltd. Chengdu, China *08/2018*

Inspection Division of R&D Department

Intern

- Learned the headspace sampling technique of gas chromatograph
- Evaluated and analyzed the intermediate precision, retrieving rate and repeatability of the acrylonitrile, divinylbenzene and chlorobenzene in residual solvent
- Tested the content of N-N-dimethylformamide in the abiraterone residual solvent.

PROFESSIONAL SKILLS

- **Microbiology & Molecular biology:** Cell/ bacteria culture, Western Blot, PCR, RT-PCR, flow cytometry, Gibson assembly, double digestion, DNA ligation, preparation of electrocompetent cell and heat-shock competent cell, chemical transformation, electroporation, in-frame gene knock-out/ knock-in in Gram-negative bacteria, Tn5-based library construction, transfection.
- **Biochemistry & Immunology:** Protein expression and purification, immobilized metal affinity chromatography (IMAC), polyclonal antisera production, HPLC, gas chromatograph, MTT assay, ELISA, H&E staining, immunofluorescence.
- **Animal experiments:** Oral inoculation of mice, certificated for JHU Animal Exposure Surveillance Program.
- **Bioinformatics & Statistics:** Python, Matlab, SnapGene, Graphpad Prism, Minitab, RasMol, RasWin, BioEdit, Primer Premier, Cytoscape, ChemDraw.

SPECIAL INTERESTS & HOBBIES

- **Arts:** Traditional Chinese painting, Chinese calligraphy, playing double bass, playing Chinese zither
- **Sports:** Playing volleyball, playing table tennis, swimming, jogging

REFERENCE

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